ISOFORMS OF STRACH BRANCHING ENZYME II (SBE-IIA AND SBE-IIB) FROM WHEAT

Field of the Invention

This invention relates generally to plant starch compositions, and concerns novel nucleotide sequences; polypeptides encoded thereby; vectors and host cells and host organisms comprising one or more of the novel sequences; a method of altering one or more characteristics of a plant; a plant having altered characteristics; starch obtained from such plants; and uses of the starch.

Background to the Invention

The majority of developments in cereal science in the recent past have concentrated primarily on the functionality of the gluten protein sub-units and their role in bakery systems. This has been greatly facilitated by the abundance of natural variation between cultivators for the gluten protein sub-unit components.

In contrast, although flour from commercially grown wheat varieties contains approximately 75-85% starch, the role of starch from a breeding perspective has been overlooked; this is largely due to the difficulty of measuring differences in starch structure. Of the limited amount of work that has been carried out however, there appears to be a lack of natural variation between different wheat cultivars. With the advent of recombinant DNA and gene transfer technologies it is now possible to create new variation *in planta*, therefore directly modifying starch composition in wheat becomes a realistic target.

Starch is the major form of carbon reserve in plants, constituting 50% or more of the dry weight of many storage organs, e.g. tubers, seeds of cereals. Starch is used in numerous food and industrial applications. In many cases, however, it is necessary to modify the native starches, via chemical or physical means, in order to produce distinct properties to

suit particular applications. It would be highly desirable to be able to produce starches with the required properties directly in the plant, thereby removing the need for additional modification. To achieve this via genetic engineering requires knowledge of the metabolic pathway of starch biosynthesis. This includes characterisation of genes and encoded gene products which catalyse the synthesis of starch. Knowledge about the regulation of starch biosynthesis raises the possibility of "re-programming" biosynthetic pathways to create starches with novel properties that could have new commercial applications.

The most significant property of starch derives from the ability of the native granular form to lose its order and to swell and absorb water upon suitable treatment, thereby conferring viscosity and texture, in a process known as gelatinisation. Gelatinisation has been defined (W A Atwell *et al*, 1988) as "... the collapse (disruption) of molecular orders within the starch granule manifested in irreversible changes in properties such as granular swelling, native crystallite melting, loss of birefringence, and starch solubilisation. The point of initial gelatinisation and the range over which it occurs is governed by starch concentration, method of observation, granule type, and heterogeneities within the granule population under observation".

14 molecules of water per molecule of anhydrous glucose, i.e. a minimum of 75% water, are necessary for full starch gelatinisation (Donovan, 1979). Starch gelatinisation is usually caused by heat, but can be caused by physical damage and some chaotropic agents, mainly dimethylsulphoxide (DMSO), urea, calcium chloride, strong base and acid.

The various events taking place during gelatinisation can be followed by various methods, including birefringence, X-ray diffraction, differential scanning calorimetry (DSC), ^{13}C NMR. Swelling can be monitored by various methods, particularly rheology.

Differential scanning calorimetry (DSC) is a destructive method which records an endothermic event on heating of granules, generally thought to measure the temperature and the endothermic energy (delta H) required for the melting of the native crystallites. Starch gelatinisation temperature is independent of water content above 75% water (described as excess water), but increases when water is limited (Donovan, 1979).

The rate and extent of starch granule swelling upon heating dictate the type of viscosity development of aqueous starch suspensions on heating. Swelling behaviour is therefore of utmost technological importance. Viscosity increase on heating can be conveniently measured by a Brabender amylograph (Brabender is a Trade Mark) (Kennedy and Cabalda, 1991) or using a Rapid Visco analyser (Rapid Visco is a Trade Mark from Newport Scientific, Australia). Figure 1 is a typical viscoamylgraph profile for wheat starch, produced in this way, showing changes in starch during and after cooking. As starch granules swell on uptake of water, in a process known as pasting, their phase volume increases, causing an increase in viscosity. The onset of pasting is indicated at A in Figure 1. Peak viscosity, indicated at B in Figure 1, is achieved when maximum phase volume is reached. Shear will then disrupt/cause fragmentation of the swollen granules, causing the viscosity to decrease. Complete dispersion is indicated at C in Figure 1. This has been confirmed by an oscillatory rheology study of starch pastes at various stages of the viscosity profile (Svegmark and Hermansson, 1990). The viscosity onset temperature and peak viscosity are indicative of the initiation and extent of swelling, respectively. On cooling, leached amylose forms a network in a process involving reassociation of molecules, or retrogradation, causing an increase in viscosity as indicated at D in Figure 1. Retrogradation (or set-back) viscosity is therefore indicative of the amount of amylose leached out of the granules.

The properties of wheat starch are useful in a large number of applications and also non-food (paper, textiles, adhesives etc.) applications. However, for many applications, properties are not optimum and various chemical and physical modifications well known in the art are undertaken in order to improve useful properties. Two types of property manipulation which would be of use are: the controlled alteration of gelatinisation and pasting temperatures; and starches which suffer less granular fragmentation during pasting than conventional starches.

Currently the only ways of manipulating the gelatinisation and pasting temperatures of starch are by the inclusion of additives such as sugars, polyhydroxy compounds of salts or by extensive physical or chemical pre-treatments. The reduction of granule fragmentation during pasting can be achieved either by extensive physical pre-treatments

or by chemical cross-linking. Such processes are inconvenient and inefficient. It is therefore desirable to obtain plants which produce starch which intrinsically possesses such advantageous properties.

Starch consists of two main glucose polysaccharides: amylose and amylopectin. Amylose is a generally linear polymer comprising α-1,4 linked glucose units, while amylopectin is a highly branched polymer consisting of an α-1,4 linked glucan backbone with α-1,6 linked glucan branches. In wheat endosperm amylopectin constitutes approximately 70% of the total starch content, with the balance being amylose. Amylopectin is synthesised through the concerted action of several enzymes, including soluble starch synthase(s) (SSS), starch branching enzyme(s) (SBE), starch de-branching enzyme(s) (DBE). The physical properties of starch are strongly affected by the relative abundance of amylose and amylopectin, therefore SSSs, SBEs and DBEs play a key role in determining both starch quantity and quality. As such, one approach to manipulating starch structure would be to modify the expression of the enzymes involved in starch biosynthesis in the endosperm using a transgenic approach.

SBE catalyses the formation of the α-1,6 linkages, creating branch points in the growing starch molecule, via hydrolysis of an α-1,4 linkage followed by reattachment of the released α-1,4-glucan chain to the same or another glucosyl chain. This reaction also provides a new non-reducing end for further elongation of the original α-1,4-glucan chain.

Multiple isoforms of starch branching enzyme have been described, biochemically, from a number of species including maize (Boyer and Preiss, 1978), rice (Nakamura *et al.*, 1992), pea (Smith, 1988), potato (Khoshnoodi *et al.*, 1993) and wheat (Morell *et al.*, 1997). More recently, genomic and cDNA sequences for SBE have been characterised from several species including maize (Baba *et al.*, 1991; Fisher *et al.*, 1993; Gao *et al.* 1997) pea (Burton *et al.*, 1995), potato (Kossmann *et al.*, 1991), rice (Nakamura and Yamanouchi, 1992; Mizuno *et al.*, 1993), *Arabidopsis* (Fisher *et al.*, 1996), cassava (Salehuzzaman *et al.*, 1992), and wheat (Rapellin *et al.*, 1997, Nair *et al.*, 1997, Rahman *et al.*, 1997). Sequence alignment of these SBEs revealed a high degree of sequence conservation at the amino acid level and that the SBEs may be grouped into two distinct

families, generally known as SBEI and SBEII. Further, analysis indicates that within a species there is generally of the order of 50% homology between the two families, SBEI and SBEII, while there is often greater homology within the two families between species.

Maize is unusual in that the maize SBEII family is thought to comprise two different members, known as SBEIIa and SBEIIb. There has been controversy over whether the SBEIIa and IIb enzymes are in fact a) encoded by genes at two different loci, and b) whether the genes represent different alleles at a single locus. Fisher et al (1996) and Gao et al (1997) have provided evidence that SBEIIa and SBEIIb are encoded by independent genes. However, there is no conclusive evidence that both isoforms exist together in any one maize genotype. The DNA clones for the two published gene sequences were purified from different genotypes of maize and it is thus possible that they represent different alleles of a single locus. In summary, in maize, three distinct SBE genes have been characterised to date (Baba *et al.*, 1991; Fisher *et al.*, 1993; Gao *et al.*, 1997). SBEI is distinct from SBEIIa and SBEIIb in amino acid composition, substrate specificity, kinetic properties, and immunological reactivities, whereas SBEIIa and SBEIIb are similar in these respects (Guan and Preiss, 1993; Preiss 1991; Takeda *et al.*, 1993). At the amino acid level the sequence exhibits approximately 50% homology with the SBEIIa and SBEIIb sequences, whereas SBEIIa and SBEIIb exhibit approximately 80% homology to each other.

Prior to the present invention, maize was unique in having SBEIIa- and SBEIIb-type enzymes. Although *Arabidopsis* has two SBEII family members, the sub-division in *Arabidopsis* does not appear to conform to that seen in maize: the *Arabidopsis* sub-family members do not obviously fall into the IIa and IIb categories as do the maize sequences. Both of the *Arabidopsis* SBEII genes have similar levels of homology to both the maize SBEII genes, SBEIIa and SBEIIb, but the similarities are not sufficient to be able to place the *Arabidopsis* genes into the same SBEIIa and SBEIIb categories as for maize. Indeed, the data, if anything, suggests that the *Arabidopsis* SBEII genes do not fall into the maize IIa and IIb categories. For barley, two forms of SBEII had been partly characterised. Although these have been called SBEIIa and SBEIIb, only a very limited amount of sequence information had been published (Sun *et al*, 1995) and it was not possible to infer

or conclude that these forms correspond to the IIa and IIb categories of maize. In fact, based on the available barley sequence information both of the barley SBEII sequences (SBEIIa and SBEIIb) would appear to show greater homology to maize SBEIIa than to maize SBEIIb.

For all other plant species for which SBEII sequences have been identified and published, including potato, pea, rice, cassava, wheat and barley, no sub-division of the SBEII family comparable to the SBEIIa and SBEIIb division of maize has been made.

Studies of purified SBEI and SBEII demonstrate that these isoforms differ in their specificity for a substrate with respect to both chain length and degree of branching. In maize, SBEI and SBEII show distinct branching activities *in vitro*, with SBEI showing a higher rate of branching of an amylose substrate when compared to SBEII whereas both SBEIIa and IIb show higher rates of branching than SBEI when acting upon an amylopectin substrate (Guan and Preiss, 1993). Furthermore, maize SBEI preferentially transfers longer glucan chains (average chain length = 24) than SBEII (average chain length = 21(IIa) and 22(IIb)) (Takeda *et al.*, 1993). A similar observation has been reported for SBEI and SBEII isoforms from wheat and pea (Morell *et al.*, 1997; Smith, 1988). Mutational studies in maize, rice and pea demonstrate that high amylose mutants in each case are deficient in the branching enzyme activity analogous to maize SBEII (Martin and Smith, 1995; Morell *et al.*, 1995). However, the linkage between the biochemical observations and the genetic evidence suggesting the differences in the roles remains unclear.

The present invention is based on the unexpected discovery of a novel class of SBEII genes in wheat, referred to herein as SBEII-1. The novel SBEII-1 gene sequence has strong homology with the maize SBEIIb gene. The wheat SBEII-1 genes are thought to be functionally equivalent to the maize SBEIIb gene, and on this basis it is believed that manipulation of the wheat SBEII-1 gene is likely to influence starch properties including starch gelatinisation temperature, in a manner analogous to manipulation of the maize SBEIIb gene as described in WO 97/22703.

In summary, although two different SBEII gene sequences are known from maize, Arabidopsis and barley, as discussed above, prior to the present invention there was no reason to expect that wheat would show a similar sub-division of SBEII genes as is seen for maize. The two Arabidopsis SBEII genes show a different sub-division, and prior to the present invention there was insufficient evidence to determine whether the two barley SBEII sequences belonged to the maize-type sub-division. That is, prior to the present invention there was no reason to expect that wheat would have two similar SBEII members comparable to those of maize. Subsequent to the present invention Sun et al (1998) have presented data which indicates that the barley sequences do indeed sub-divide in a similar manner to the maize SBEIIa and IIb sequences and the wheat SBEII-2 and SBEII-1 sequences discussed in this document.

The present inventors have used the high degree of sequence conservation between several SBE gene sequences to design oligonucleotide primers to motifs which are specific to either SBEI or SBEII families and have used these primers to amplify cDNA sequences from developing endosperm of wheat.

When this work was started, a single partial length wheat SBE cDNA clone had been reported (Mousley, 1994). Multiple sequence alignment of this wheat SBE sequence with other published SBE sequences from a number of plant species revealed a number of motifs which were highly conserved. Oligonucleotide primers designed to be complementary to these motifs were used to clone 3' partial length cDNA clones of wheat SBEII. Alignment of the cDNA clone sequences indicated that the clones could be divided into two classes, which the inventors have designated SBEII-1 and SBEII-2, which showed greater than 90% similarity to members within a class but only 60% similarity between classes. Significantly, comparison between representative sequences from each class with previously identified wheat SBEII clones, pWBE6 (Mousley, 1994) and SBEII (Nair *et al.*, 1997), showed that each appear to be homologues of the SBEII-2 class. The cloning of a wheat SBEII-1 cDNA is novel.

Summary of the Invention

In one aspect the invention provides a nucleotide sequence encoding substantially the amino acid sequence shown in Figure 10 (SEQ ID No: 2) or a functional equivalent of said nucleotide sequence.

The term functional equivalent is used in this context to encompass those sequences which differ in their nucleotide composition to that shown in Figure 10 (SEQ ID No: 1) but which, by virtue of the degeneracy of the genetic code, encode polypeptides having identical or substantially identical amino acid sequences. It is intended that the term should generally apply to sequences which are sufficiently homologous to the sequence of the invention that they can hybridise to the complement thereof under stringent hybridisation conditions (eg as described by Sambrook et al 1989, ie washing with 0.1xSSC, 0.5% SDS at 68°C); such equivalents will preferably possess at least 86%, more preferably at least 90%, and most preferably at least 95%, sequence homology (ie sequence similarity) with the sequence of the invention. Sequence homology is suitably determined using the 'MEGALIGN' program of the software package DNAStar (MEGALIGN and DNAStar are Trade Marks). It will be apparent to those skilled in the art that the nucleotide sequence of the invention may also find useful application when present as an "antisense" sequence. Accordingly, functionally equivalent sequences will also include those sequences which can hybridise, under stringent hybridisation conditions, to the sequence of the invention (rather than the complement thereof). Such "antisense" equivalents will preferably possess at least 86%, more preferably at least 90%, and most preferably 95% sequence homology with the complement of the sequence of the invention.

In another aspect, the invention provides a nucleotide sequence comprising substantially the sequence of B2 shown in Figure 3 (SEQ ID No: 3), or a functional equivalent thereof.

In a further aspect, the invention provides a nucleotide sequence comprising substantially the sequence of B4 shown in Figure 3 (SEQ ID No: 4), or a functional equivalent thereof.

Another aspect of the invention provides a nucleotide sequence comprising substantially

the sequence of B10 shown in Figure 3 (SEQ ID No: 5), or a functional equivalent thereof.

Yet a further aspect of the invention provides a nucleotide sequence comprising substantially the sequence of B1 shown in Figure 3 (SEQ ID No: 6), or a functional equivalent thereof.

In another aspect the invention provides a nucleotide sequence encoding substantially the amino acid sequence of B6 shown in Figure 4 (SEQ ID No: 7), or a functional equivalent thereof.

The term functional equivalent in this context has the same general meaning as discussed above, although equivalents for B2, B4, B10 and B6 will preferably possess at least 90%, more preferably at least 95%, sequence homology with the relevant sequence of the invention, while equivalents for B1 will preferably possess at least 97% sequence homology with the sequence of the invention.

The sequences of the invention are part of novel wheat SBEII genes, with B1 being a novel subclass of the known class of SBEII genes, referred to herein as SBEII-2, with the novel subclass being called SBEII-2B. The remaining sequences are all of a completely new class of wheat SBEII genes, referred to herein as SBEII-1. The sequences have been found to fall into 3 sub-classes, to be discussed below.

The novel wheat SBEII-1 genes that are the subject of this invention have strong sequence homology with the maize SBEIIb gene. The wheat SBEII-1 genes are thought to have similar functional properties to the maize SBEIIb gene. On this basis it is expected that by genetic manipulation of the wheat SBEII-1 gene it will be possible to influence properties of starch produced by a plant, including the gelatinisation temperature and rheological properties of starch, in a manner analogous to manipulation of the maize SBEIIb gene described in WO 97/22703. The content of WO 97/22703 is incorporated herein by reference.

The present invention also includes within its scope a portion of any of the above sequences, comprising at least 500 base pairs and having at least 90% sequence homology to the corresponding portion of the sequence from which it is derived.

Although the coding sequences of the novel wheat SBEII-1 genes have strong sequence homology with the maize SBEIIb gene, there is much greater divergence in the 3' untranslated parts of the sequences, with a maximum of 31.8% homology between the 3' untranslated sequences of wheat SBEII-1 and maize SBEIIb as is apparent from Figure 8.

In another aspect the invention thus provides a nucleotide sequence comprising substantially the sequence shown in Figure 5 (SEQ ID No: 8), Figure 6 (SEQ ID No: 9) or Figure 7 (SEQ ID No: 10), or a functional equivalent thereof.

The term functional equivalent in this context has the same general meaning as discussed above, but with equivalents preferably at least 32%, more preferably at least 40%, 50%, 60%, 70%, 80% or 90% sequence homology with the sequence of the relevant Figure.

It is thought such 3' untranslated sequences may be useful, both in sense and antisense function, in manipulation of starch properties by affecting SBE expression in plants, as will be discussed below.

The sequence may include further nucleotides at the 5' or 3' end. For example, for ease of expression, the sequence desirably also comprises an in-frame ATG start code, and may also encode a leader sequence.

The invention also covers a nucleic acid construct comprising a nucleotide sequence or portion thereof in accordance with the invention conveniently operably linked, in sense or antisense orientation, to a promoter sequence.

Also included within the scope of the invention is amino acid sequence encoded by any of the nucleotide sequences of the invention.

The invention also provides vectors, particularly expression vectors, comprising the nucleotide sequence of the invention. The vector will typically comprise a promoter and one or more regulatory signals of the type well known to those skilled in the art. The invention also includes provision of cells transformed (which term encompasses transduction and transfection) with a vector comprising the nucleotide sequence of the invention.

Nucleotide sequences in accordance with the invention may be introduced into plants, particularly but not exclusively wheat plants, and it is expected that this can be used to affect expression of SBE in the plant and hence affect the properties of starch produced by the plant. In particular, use of sequences in antisense orientation is expected to reduce or suppress enzyme expression. Additionally, it has recently been demonstrated in other experimental systems that "sense suppression" can also occur (i.e. expression of an introduced sequence operably linked in the sense orientation can interfere, by some unknown mechanism, with the expression of the native gene), as described by Matzke & Matzke 1995. Any one of the methods mentioned by Matzke & Matzke could, in theory, be used to affect the expression in a host of a homologous SBE gene.

It is believed that antisense methods are mainly operable by the production of antisense mRNA which hybridises to the sense mRNA, preventing its translation into functional polypeptide, possibly by causing the hybrid RNA to be degraded (e.g. Sheehy *et al.*, 1988; Van der Krol *et al.*,). Sense suppression also requires homology between the introduced sequence and the target gene, but the exact mechanism is unclear. It is apparent however that, in relation to both antisense and sense suppression, neither a full length nucleotide sequence, nor a "native" sequence is essential. Preferably the "effective portion" used in the method will comprise at least one third of the full length sequence, but by simply trial and error other fragments (smaller or larger) may be found which are functional in altering the characteristics of the plant.

Thus, in a further aspect the invention provides a method of altering the characteristics of a plant, comprising introducing into the plant an effective portion of the sequence of the invention operably linked to a suitable promoter active in the plant so as to affect

expression of a gene present in the plant. Conveniently the sequence will be linked in the antisense orientation to the promoter. Preferably the plant is a wheat plant. Conveniently, the characteristic altered relates to the starch content and/or starch composition of the plant (i.e. amount and/or type of starch present in the plant). Preferably the method of altering the characteristics of the plant will also comprise the introduction of one or more further sequences, in addition to an effective portion of the sequence of the invention. The introduced sequence of the invention and the one or more further sequences (which may be sense or antisense sequences) may be operably linked to a single promoter (which would ensure both sequences were transcribed at essentially the same time), or may be operably linked to separate promoters (which may be necessary for optimal expression). Where separate promoters are employed they may be identical to each other or different. Suitable promoters are well known to those skilled in the art and include both constitutive and inducible types. Examples include the CaMV 35S promoter (e.g. single or tandem repeat) and the ubiquitin promoter. Advantageously the promoter will be tissue-specific. Desirably the promoter will cause expression of the operably linked sequence at substantial levels only in the tissue of the plant where starch synthesis and/or starch storage mainly occurs.

The sequence of the invention, and the one or more further sequences if desired, can be introduced into the plant by any one of a number of well-known techniques (e.g. *Agrobacterium*-mediated transformation, or by "biolistic" methods). The sequences are likely to be most effective in affecting SBE activity in wheat plants, but theoretically could be introduced into any plant. Desirable examples include pea, tomato, maize, rice, barley, sweet potato and cassava plants. Preferably the plant will comprise a natural gene encoding an SBE molecule which exhibits reasonable homology with the introduced nucleic acid sequence of the invention.

In another aspect, the invention provides a plant cell, or a plant or the progeny thereof, which has been altered by the method defined above. The progeny of the altered plant may be obtained, for example, by vegetative propagation, or by crossing the altered plant and reserving the seed so obtained. The invention also covers parts of the altered plant, such as storage organs. Conveniently, for example, the invention covers grain comprising

altered starch, said grain being obtained from an altered plant or the progeny thereof. Grain obtained from altered plants (or the progeny thereof) will be particularly useful materials in certain industrial applications and for the preparation and/or processing of foodstuffs and may be used, for example, in bakery products.

In particular relation to wheat plants, the invention provides a wheat plant or part thereof which, in its wild type possesses an effective SBEII-1 gene, but which plant has been altered such that there is either reduced, increased or no effective expression of an SBEII-1 polypeptide within the cells of at least part of the plant. The plant may have been altered by the method defined above, or may have been selected by conventional breeding to be deleted for the SBEII-1 gene, the presence or absence of which can be readily determined by screening samples of the plants with a nucleic acid probe or antibody specific for the wheat gene or gene product respectively.

The invention also provides starch extracted from a plant altered by the method defined above, or from the progeny of such a plant, the starch having altered properties compared to starch extracted from equivalent, but unaltered, plants. The invention further provides a method of making altered starch, comprising altering a plant by the method defined above and extracting therefrom starch having altered properties compared to starch extracted from equivalent, but unaltered, plants. It is believed that use of nucleotide sequences in accordance with the invention will enable the production of starches, particularly wheat starches, having a wide variety of novel properties. For example, it may be anticipated that plants altered to give a reduction in SBEII activity will give rise to a starch with a relatively higher proportion of amylose and a lower proportion of amylopectin compared with that from unaltered plants.

In particular the invention provides the following: a plant (especially a wheat plant) altered by the method defined above, containing starch which, when extracted from the plant, has an elevated gelatinisation onset and/or peak temperature as measured by DSC, compared to starch extracted from a similar, but unaltered, plant; a plant (especially a wheat plant) altered by the method defined above, containing starch which, when extracted from the plant, has a elevated gelatinisation onset temperature (conveniently elevated by at least

3°C, possibly by at least 7°C, by at least 12°C or possibly even by 15 to 25°C) as measured by DSC compared to starch extracted from a similar, but unaltered plant; a plant (especially a wheat plant) altered by the method defined above, particularly to reduce expression of SBEII-1 polypeptide, containing starch which, when extracted from a plant, has a higher amylose:amylopectin ratio compared to starch extracted from a similar, but unaltered plant.

The present invention particularly covers starch extracted from a plant altered by the method of the invention, particularly starch having an increased gelatinisation temperature. Such starch is useful, eg in bakery products, having particular benefits in certain situations, and the invention also covers products, particularly bakery products, made from such starch. The invention also covers starch extracted from a plant altered by the method of the invention and having an increased amylose:amylopectin ratio.

The invention will be further described, by way of illustration, in the following Examples and with reference to the accompanying drawings, in which:

A BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph of viscosity versus time, showing a viscoamylgraph profile for wheat starch during and after cooking;

Figure 2 shows alignment amino acid sequence data of C terminal portions of various known starch branching enzymes (SEQ ID Nos: 12 to 25), obtained from the European Molecular Biology Laboratory (EMBL) database, and for a novel wheat SBEII-1 sequence of the invention (OsbeII-1ALL) (SEQ ID No: 11) from clone 5A1, with consensus residues highlighted;

Figure 2a is a residue weight table showing the percent similarity and percent divergence of the sequences shown in Figure 2;

Figure 3 shows aligned DNA sequence data for various recombinant clones (B2, B4, B10, A2, B1, B11) (SEQ ID Nos: 3, 4, 5, 26, 6, 27 respectively) containing wheat starch branching enzyme genes, representing two SBE classes, SBEII-1 and SBEII-2, each of

which includes three subclasses A, B and C, with residues differing from the consensus (majority) (SEQ ID No: 53) highlighted;

Figure 3a is a residue weight table showing the percent similarity and percent divergence of the sequences shown in Figure 3;

Figure 4 is an alignment of predicted amino acid sequences for clones B6 (wheat SBEII-1) (SEQ ID No: 7) and B11 (wheat SBEII-2) (SEQ ID No: 28) against the corresponding regions of the maize SBEIIa (SEQ ID No: 29) and SBEIIb (SEQ ID No: 30) amino acid sequences, with residues differing from those of maize SBEIIb highlighted;

Figure 4a is a residue weight table showing the percent similarity and percent divergence of the sequences shown in Figure 4;

Figure 5 shows the 3' untranslated DNA sequence of clone B2 (SEQ ID No: 8) (wheat SBEII-1, sub-class A);

Figure 6 shows the 3' untranslated DNA sequence of clone B10 (SEQ ID No: 9) (wheat SBEII-1, sub-class B);

Figure 7 shows the 3' untranslated DNA sequence of clone B4 (SEQ ID No: 10) (wheat SBEII-1, sub-class C);

Figure 8 shows aligned DNA sequence data for the 3' untranslated region of clones B10 (SEQ ID No: 9), B2 (SEQ ID No: 8) and B4 (SEQ ID No: 10) and maize SBEIIb (ZMSBE2b) (SEQ ID No: 31), with residues differing from those of the B10 sequence highlighted;

Figure 8a is a residue weight table showing the percent similarity and percent divergence of the sequences shown in Figure 8;

Figures 9a and 9b show hybridisation of clone B1 (SBEII-2) and clone B2 (SBEII-1),

respectively, to HindIII-digested genomic DNA of Chinese Spring wheat nullisomic-tetrasomic lines;

Figure 10 shows the DNA (SEQ ID No: 1) and predicted amino acid sequence (SEQ ID No: 2) of part of SBEII-1 clone 5A1;

Figure 11 shows aligned amino acid sequence data for the wheat SBEII-1 sequence of the invention, from clone 5AI (OsbeII-1ALL) (SEQ ID No: 11), wheat SBEI-D2 (SEQ ID No: 32) of Rahman *et al* 1997 (TASBEID2), wheat SBE1 of Rapellin *et al* 1997 (SEQ ID No: 33) (TASBEI) and wheat SBEII-2 of Nair *et al* 1997 (SEQ ID No: 34) (wheat SBEII-2), with residues exactly matching the consensus (majority) (SEQ ID No: 54) highlighted;

Figure 11a is a residue weight table showing the percent similarity and percent divergence of the sequences shown in Figure 11;

Figure 12 illustrates northern blotting of wheat grains harvested at various different intervals after anthesis and probed with SBEII-1 and SBEII-2 fragments;

Figure 13 is a restriction map of plasmid pWxGS+;

Figure 13a shows the sequence (SEQ ID No: 55) of the promoter (HindIII-BamH1 fragment) in pWxGS+;

Figure 14 is a restriction map of plasmid pSRWXGUS1;

Figure 15 is a restriction map of plasmid pVTWXGUS2;

Figure 16 is a restriction map of plasmid pPBI-97-2;

Figure 17 is a restriction map of plasmid pSR97-26A-;

Figure 18 is a restriction map of plasmid pSR97-29A-;

Figure 19 is a restriction map of plasmid pSR97-50A-;

Figure 20 is a restriction map of plasmid pSR97-53A-;

Figure 21 is a restriction map of plasmid p97-2C;

Figure 22 is a restriction map of plasmid p97-2CWT1;

Figure 23 is a restriction map of plasmid pSC98-1;

Figure 24 is a restriction map of plasmid pSC98-2;

Figure 25 is a restriction map of plasmid pUNI;

Figure 26 shows the DNA sequence of the NptII Sac1 fragment of pUNI (SEQ ID No: 35); and

Figure 27 is a restriction map of plasmid pUSN99-1;

Figure 28 is a restriction map of plasmid pUSN99-2;

Figure 29 is a partial restriction map of the predicted sequence (SEQ ID No: 52) of a cloned fragment of p97-U3;

Figure 30 is a restriction map of plasmid pPBI96-36;

Figure 31 is a restriction map of plasmid p97-dUG1;

Figure 32 is a restriction map of plasmid p97-2BdUN1;

Figure 33 is a schematic illustration of a particle bombardment chamber (not to scale);

Figure 34 shows histochemical localisation of Ubi-GUS expression in seed (panel A), stem (panel B), floral (panel C) and leaf tissues (panel D) of wheat transformed with plasmid pAHC25;

Figure 35 is a Southern blot of 26 progeny plants of transformant BW119 which had been transformed with pAHC25.

Figure 36 shows histochemical localisation of waxy-GUS expression in endosperm tissue of two independent transgenic wheat lines (in panels A and B) transformed with the plasmid pWxGS+; and

Figure 37 is a Southern blot of genomic DNA of putative primary transformants digested with Sac1 and probed with the 1kb Sac1 SBEII-1 probe.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Examples

Amplification and characterisation of two classes of SBEII cDNA clones

A PCR based cloning strategy was devised for isolating starch branching enzymes from wheat using conserved domains within the known cloned gene sequences. Starch branching enzymes have been cloned from a number of plant species and Figure 2 shows amino acid sequence data, obtained from the European Molecular Biology Laboratory (EMBL) nucleotide database for various known starch branching enzymes as follows:-

Wheat SBEII-2 for *Triticum aestivum* (SEQ ID No: 12)
ZM SBE2a (maize) for *Zea mays* (SEQ ID No: 13)
ZM SBE2b (maize) for *Zea mays* (SEQ ID No: 14)
Barley SBEIIa (SEQ ID No: 15)
Barley SBEIIb (SEQ ID No: 16)
RICBCE3 (rice SBEII type enzyme) for *Oryza sativa* (SEQ ID No: 17)

RICESBE-1/97 (as above, including transit peptide sequence) (SEQ ID No: 18)
PSSBEIGEN (pea SBEI, which is in fact an SBEII- type sequence) for *Pisum sativum* (SEQ ID No: 19)
STSBE (potato SBEI type) for *Solanum tuberosum* (SEQ ID No: 20)
TASBEI (wheat SBEI-2) for *Triticum aestivum* (SEQ ID No: 21)
TASBEI D2 (SEQ ID No: 22)
ZMSBEI (maize SBEI) for *Zea mays* (SEQ ID No: 23)
RICBEI (rice SBEI) for *Oryza sativa* (SEQ ID No: 24)
PSSBEIIGN (pea SBEII, which is in fact an SBEI-type sequence) for *Pisum sativum* (SEQ ID No: 25)

Figure 2 also shows sequence information for a novel wheat SBEII-1 sequence of the invention, identified as OsbeII-1ALL (SEQ ID No: 11).

The alignment report of Figure 2, and also Figures 3, 4, 8 and 11, was prepared using Clustal method, with PAM 250 residue weight table for amino acid sequences and weighted residue weight table for DNA sequences. Sequence pair distances expressed as % similarity shown in Figures 2A and 3A, 4A, 8A and 11A are determined using a 'MEGALIGN' program of DNAStar software, and correspond to sequence homology percentages as specified above.

Alignment of the sequences shown in Figure 2 reveals several domains which are highly conserved. One such domain, MDKDMYD (SEQ ID No: 36), was almost completely conserved and it was assumed that this domain would also be present in wheat starch branching enzyme genes. This motif was chosen as a target for an oligonucleotide sense primer (SBEA). 3'RACE PCR was carried out on endosperm first strand cDNA using the primers Ro and SBE A.

Two populations of PCR products of approximately 1kb and 1.2Kb were cloned into the plasmid vector pT7Blue (Novagen). Plasmid DNA from 36 putative recombinant clones was purified and the insert size estimated by restriction analysis. Fifteen clones harbouring inserts of between approximately 1Kb and 1.2Kb were selected for sequencing.

Alignment of the sequence data obtained, using the MEGALIGN program of DNAStar, indicated that the 15 selected clones could be divided on the basis of degrees of homology into two different classes, which we have designated SBEII-1 and SBEII-2. Furthermore, both the SBEII-1 and SBEII-2 classes may each be further subdivided into three sub-classes, based on sequence differences (Table 1). It is thought the sub-division into three sub-classes probably arises because wheat comprises three homoeologous genomes.

Table 1

Class	Sub-Class	Clone Number
SBEII-1	A	B2, B5, B6, B7, B12
SBEII-1	B	B10
SBEII-1	C	A1, A13, B4
SBEII-2	A	B11
SBEII-2	B	B1, B9
SBEII-2	C	A2, C5

Comparison between sequences within either of the SBEII-1 or SBEII-2 classes showed between 90 and 96.8% similarity. In contrast, sequence similarity between representatives of SBEII-1 and SBEII-2 classes only display between 58.8 and 60.0% homology in the region of comparison (Figures 3 and 3a).

Furthermore, we have compared representative sequences from each SBEII-1 and SBEII-2 class with the previously reported wheat SBEII clones, pWBE6 (Mousley, 1994) and the very recently published SBEII (Nair *et al.*, 1997). The results showed that each of the previously isolated SBEII clones are highly homologous (>90%) to our SBEII-2 class (data not shown). Significantly, neither of the previously reported wheat sequences showed high homology to our SBEII-1 sequence. The isolation and characterisation of three forms of SBEII-1 (SBEII-1, sub-classes A, B & C) is novel. The SBEII-2 sub-class B is also novel, sub-classes A and C corresponding to the sequences previously disclosed by Mousley (1994) and Nair *et al* (1997) respectively.

Alignment of the predicted amino acid sequences from representative clones, B6 and B11 of the wheat SBEII-1 and SBEII-2 sequences (respectively) against the corresponding regions of the maize SBEIIa and SBEIIb amino acid sequences (Figure 4 and 4a) indicate that the wheat SBEII-1 sequence (clone B6) is more similar to the maize SBEIIb sequence (88.7% similarity) than to the wheat SBEII-2 sequence and the maize SBEIIa sequence (82.2% & 82.6% similarity respectively) and similarly that the wheat SBEII-2 sequence is more similar to the maize SBEIIa sequence (86.9% similarity) than to the wheat SBEII-1 and maize SBEIIb sequences (82.2% and 81.7% similarity respectively). We thus hypothesise that the wheat SBEII-1 is phylogenetically more related to the maize SBEIIb and that the wheat SBEII-2 is phylogenetically related to the maize SBEIIa sequences and that the corresponding wheat and maize sequences are likely to exhibit similar functional properties.

While the coding sequences of clones B2, B10 and B4 have strong sequence homology to the maize SBEIIb gene, there is much greater divergence in the 3' untranslated parts of the sequences. Figure 5, 6 and 7 show the 3' untranslated sequences of clones B2, B10 and B4, respectively, and Figure 8 compares these sequences with the corresponding sequence of maize SBEIIb.

Considering matters in more detail, experimental details were as follows.

Plant material

Triticum aestivum cultivar Rialto was grown in a glass house under supplementary lighting and temperature control to maintain a 16 hour day-length at 18+/-1°C.

Recombinant DNA manipulations and sequencing

Standard procedures were performed essentially according to Sambrook *et al.*, (1989). DNA sequencing was performed on an ABI automated sequencer and sequences analysed using DNASTAR software for Macintosh.

RNA isolation for cDNA cloning

RNA was extracted from *Triticum aestivum* cultivar Rialto endosperm, using a Purescript RNA isolation kit (Flowgen) essentially according to the manufacturers recommendations. Briefly, endosperm tissue was frozen in liquid nitrogen and ground, for 2 min, to a fine powder using a dismembrenator (Braun Biotech International). The ground tissue was stored in liquid nitrogen prior to extraction. Approx. 100mg of ground tissue was transferred to a 1.5ml microcentrifuge tube and 1.2ml of 'Lysis buffer' was added to the tissue before mixing by inversion and placing on ice for 10 minutes. Protein and DNA were precipitated from the cell lysate by adding 0.4ml of 'Protein-DNA Precipitation Solution' and mixing by inversion before centrifuging at 13,000 x *g* at 4°C for 20 minutes. The supernatant was divided between two fresh 1.5ml tubes each containing 600μl of *iso*-propanol. The RNA precipitate was pelleted by centrifugation at 13,000 x *g* at 4°C for 10 minutes, the supernatant was discarded and the pellets washed with 70% ethanol by inverting the tube several times. The ethanol was discarded and the pellet air dried for 15-20 minutes before the RNA was resuspended in 7.5ml of 'RNA Hydration Solution'.

Preparation of wheat endosperm cDNA pool

Wheat endosperm cDNA pool was prepared from total RNA, extracted as described above, using Superscript™ reverse transcriptase (Life Technologies) essentially according to manufacturers instructions. Briefly, five microgrammes of RNA, 10pMol RoRidT17 [AAGGATCCGTCGACATCGATAATACGACTCACTATAGGGA(T17)] (SEQ ID No: 37) and sterile distilled water to a reaction volume of 12μl, in a 500μl microcentrifuge tube, was heated to 70°C for 10 minutes before being quick chilled on ice. The contents of the tube were collected by brief centrifugation before adding 4μl 5x First Strand Buffer, 2μl 0.1M DTT and 1μl 10mM dNTPs and, after mixing, incubating at 42°C for 2 min. 1μl of Superscript™ was added and, after mixing, incubation continued for 1 hour. The reaction was inactivated by heating to 70°C for 15 min. 150μl of $T_{10}E_1$ was added to the reaction mix and the resulting cDNA pool was used as a template for amplification in PCR.

PCR amplification of SBEII sequences from endosperm cDNA pool

SBEII sequences were amplified from the endosperm cDNA pool using primers Ro [AAGGATCCGTCGACATC] (SEQ ID No: 38), which is complementary to the Ro region of the RoRidT17 primer used to synthesise the cDNA pool, and the SBEII specific primer, SBEA [ATGGACAAGGATATGTATGA] (SEQ ID No: 39). SBEA was designed to be homologous to the MDKDMYD (SEQ ID No: 36) motif which is situated approx. 1kb from the 3'end of the mature peptide coding sequence. PCR was carried out in a 50μl reaction, comprising 5μl of the cDNA pool, 25pmol Ro, 50pmol SBEA, 5μl 5x Taq buffer, 4μl 25mM Mg^{2+}, 0.5μl 20mM dNTPs, and 1.25u Taq polymerase. All of the reaction components were mixed, except for the Taq polymerase, before being pre-heated to 94°C for 7 min and then cooled to 75°C for 5 min. Whilst the reaction mixtures were held at 75°C the Taq polymerase was added and, after mixing well, the reactions were thermocycled at (94°C-30sec, 50°C-30sec, 72°C-1min) x 30 cycles, followed by a final 10 min extension step at 72°C.

PCR products were purified by phenol/chloroform and chloroform extraction before ligation with pT7 Blue (Novagen) according to manufacturers recommendations. Putative SBE clones were initially characterised by standard plasmid DNA purification methods and restriction digestion. Representative clones harbouring a range of different sized inserts were selected for sequencing.

Chromosomal location of SBE genes in wheat

The Chinese Spring wheat nullisomic-tetrasomic lines as described in Sears (1966) were used for assignment of the SBE sequences chromosome locations. Ditelosomic lines (Sears, 1966) were used to determine the chromosome arm location. The Betzes barley ditelosomic addition lines in wheat are described in Islam (1983).

The chromosomal location of the two families of SBEII sequences (SBEII-1, SBEII-2) was determined by probing wheat nulli-tetra and ditelosomic stock lines with gel-purified inserts of the various clones. Figure 9a shows the hybridisation obtained with an SBEII-2

(clone B1) probe on HindIII digested DNA. The euploid Chinese Spring gives 3 bands, one of which is missing in turn in the lines nullisomic for chromosomes 2A, 2B and 2D. The same blot was re-probed with a SBEII-1 specific probe (clone B2). This yields an entirely different hybridisation profile (Figure 9b), demonstrating the specificity of the probe used. Again bands are missing in each of the lines nullisomic for 2A, 2B and 2D. the same banding pattern was observed using the SBEII-1 clones B2 and B4. Thus the SBEII sub-family 1 and 2 gene sequences lie on the wheat group 2 set of homeologous chromosomes.

Ditelosomic addition lines were used to identify the arm location of these genes (data not shown). This revealed that the SBEII-1 and SBEII-2 sequences are both located on the long arms of the homeologous group 2 chromosomes of wheat.

Barley addition lines were used to determine whether homologous sequences are present in barley. These showed that sequences homologous to the wheat SBEII-1 and SBEII-2 sequences are located on the long arms of barley chromosome 2H.

RNA Isolation and Northern Blotting

Wheat grains were harvested at appropriate intervals and frozen in liquid Nitrogen before grinding to a fine powder using either a Braun Mikrodismembrator™ or a pestle and mortar. Total RNA was isolated using the RNAqueous™ (Ambion Inc) Kit according to the manufacturers instructions, or with the following method. Frozen powdered grain was mixed with a 10X volume of 0.2M Tris-HCl pH9, 0.4M NaCl, 25mM EDTA, 1% SDS, 1% PVPP, 0.25% Antifoam A, and 0.1M DTT. This mixture was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), the nucleic acids precipitated from the aqueous phase by the addition of 0.8 volumes of isopropanol, and the resulting pellet dissolved in H_2O. The RNA was then selectively precipitated by the addition of 1 volume of 4M LiCl, incubated at 4°C overnight, and the resulting pellet dissolved in sterile distilled H_2O. 15 μg of total RNA was electrophoresed on a 1% agarose, 2.21M Formaldehyde, 40mM MOPS pH7.0, 10mM sodium acetate, 1mM EDTA gel, in a 40mM MOPS pH7, 10mM sodium acetate, 1mM EDTA running buffer at 1

V/cm overnight. Gels were placed in a 50ng/ml solution of Ethidium Bromide in water for 30 minutes, de-stained in water for 2 hours, and visualised and photographs under UV light. The gels were then washed briefly in sterile distilled H_2O, then blotted onto HyBond N^{+TM} (Amersham International), according to standard protocols (Sambrook et al, 1989) overnight. Blots were then dismantled and air-dried before UV fixing at 312nm for 2 minutes.

Probe Isolation and Purification

5-10 μg of the plasmids pUN1 and pSR98-29 were digested with Sst1 (Life Technologies Ltd) according to the manufacturers instructions, to release fragments of approximately 0.8kb (NptII) and 1kb (SBEII-1) respectively. 5-10μg of the plasmid pVT96-54 was digested with BamH1 to release a SBEII-2 fragment of approximately 1.2kb. Digests were electrophoresed on 1% low melting point agarose gels. The gene specific fragments were excised and the DNA purified using a Wizard^{TM} Gel Purification Kit (Promega).

Probe Labelling and Hybridization

25ng of the appropriate probe (Maize Waxy promoter, NptII, Wheat SBEII-1 or Wheat SBEII-2 fragments) were radiolabelled using the Rediprime 11^{TM} system (Amersham International) using α^{32}PdCTP (Amersham International) according to manufacturers instructions. Blots were hybridized overnight at 65°C in 0.6M NaCl, 20mM Pipes, 4mM $Na_2EDTA2H_2O$, 0.2% gelatin, 0.2% Ficoll 400, 0.2% PVP-360, 10mM $Na_4P_2O_710H_2O$, 0.8% SDS, 0.5mg/ml denatured salmon sperm DNA. Post hybridization washes were carried out in 30mM NaCl, 2Mm $NaH_2PO_4.2H_2O$, 0.2mM $Na_2EDTA.2H_2O$, 0.1% SDS at room temperature for 7 minutes, then 65°C for 10 minutes. Filters were exposed to Kodak BioMax MR^{TM} (Amersham International) film at -70°C. Blots were stripped by washing in 15mM NaCl, 1mM $NaH_2PO_4.2H_2O$, 0.1mM EDTA at 90°C for 10 minutes, or until no counts above background remained.

Extension of the SBEII-1 3' sequence towards the 5'end of the mature peptide

We have exploited the sequence divergence between our wheat SBEII-1 and SBEII-2 sequences to design the SBEII-1 specific 3' primer, Sb4. This primer was used in conjunction with an SBEII specific 5' primer to extend the novel SBEII-1 sequence using a PCR-based approach.

To extend the SBEII-1 3' sequence towards the 5'end of the mature peptide, a second conserved domain was identified and an oligonucleotide sense primer, AGSBEI, designed. PCR amplification from the endosperm first strand cDNA pool was carried out using the AGSBEI-Sb4 primer pair. Separation of the amplification products by electrophoresis through a 1% (w/v) agarose gel (data not shown) showed that the reaction yielded a distinct band of approx. 2.2kb. The approx 2.2kb amplification products were excised from the gel, ligated with PT7Blue and transformed into competent Novablue *E.coli* cells. Following overnight culture, nine putative recombinant clones were selected for further analysis. Screening of each of the selected clones using vector specific primers indicated that clones 5A1, 5A2, 5A5 and 5A9 harboured inserts of the predicted size. Of these clone 5A1 (which falls in sub-class C) was selected for sequencing (Figure 10). The amino acid sequence of Figure 10 corresponds to the OsbeII-1ALL sequence of Figure 2. Although not full length the predicted open reading frame includes nucleotides 44 through to 1823 and encodes a 593 amino acid peptide. Based on similarities with the maize genes, it is estimated that this sequence is missing approximately 230 amino acids out of a predicted total of approximately 830 amino acids. On this basis, the partial sequence represents about 70% of the coding sequence. Multiple sequence alignment of this SBEII-1 sequence with recently published wheat SBEII-2 (Nair *et al.*, 1997), SBEI (Rapellin *et al.*, 1997) and SBEI-D2 (Rahman *et al.*, 1997) sequences showed that the SBEII-1 sequence has similarity indices of 69.6%, 31.2% and 46.7% to SBEII-2, SBEI and SBEI-D2 respectively (Figures 11 and 11a). This demonstrates that the SBEII-1 sequence differs from the published wheat SBE sequences, and confirms the analysis of the 3' sequence alignment (Figure 3). The increase in relative homology when compared to the values obtained following 3'sequence alignment results from the fact that the central domain of SBEs is highly conserved (Burton *et al.*, 1995; Gao *et al.*, 1997). However, it is clear

that this cloned wheat SBEII-1 sequence is significantly different from previously published wheat SBE sequences and represents a novel sequence.

Full experimental details were as follows.

SBEII-1 sequences were extended toward the 5'end of the mature peptide by amplification from the endosperm cDNA pool using the SBEII-1 specific primer Sb4 [TTTTCTTCACAACGCCCTGGG] (SEQ ID No: 40) in conjunction with the primer AGSBEI [TGTTTGGGAGATCTTCCTCCC] (SEQ ID No: 41). AGSBEI was designed to be homologous to the GVWEIFLP (SEQ ID No: 42) motif which is conserved in all known SBE sequences and is situated toward the 5'end of the mature peptide coding sequence. PCR was carried out in a 50μl reaction, comprising 5μl of the cDNA pool, 50pmol Sb4, 50pmol SBEA1, 5μl 5x Taq buffer, 4μl 25mM Mg^{2+}, 0.5μl 20mM dNTPs, and 1.25u Taq polymerase. All of the reaction components were mixed, before thermocycling at (94°C-45sec, 55°C-30sec, 72°C-1min 30sec) x 30 cycles, followed by a final 10 min extension step at 72°C. Amplification products were separated by electrophoresis through a 1%(w/v) agarose gel and specific amplification products of the predicted size were excised from the gel. The DNA was eluted from the gel slice using QIAGEN's gel extraction kit according to the manufacturers recommendations before ligation with pT7 Blue (Novagen). Ligation was carried out in a 10μl reaction volume comprising 7.5μl purified amplification product, 1μl 10x ligation buffer, 1μl pT7Blue and 0.5μl T4 DNA ligase (Amersham). The reaction components were mixed well before being placed at 4°C overnight. Following overnight incubation, half of the ligation reaction was used to transform competent Novablue *E.coli* cells (Novagen). Transformed cells were plated out onto LB plates supplemented with X-gal (40μgml^{-1}), IPTG (0.1mM), Carbenicillin (100μgml^{-1}), and Tetracycline (12.5μgml^{-1}), before placing at 37°C overnight. Putative recombinant clones were initially screened for the presence of an insert by colony PCR using the vector specific primers T7B and U19. Insert positive clones were then screened using an insert specific primer in conjunction with either T7B or U19 primers to determine the orientation of the insert within the multiple cloning site prior to sequencing.

Southern blot analysis

Southern analyses of the pre-made nulli-tetra and ditelosomic blots were carried out essentially as described in Jack *et al* (1994).

The SBEII-1 clones discussed above have been cloned into transformation vectors for transformation of wheat.

Northern blot analysis

Northern blots were prepared from total RNA from developing wheat grains of the cultivar Bobwhite. Figure 12 shows a northern blot of RNA from wheat grains of the cultivar Bobwhite grown in the glasshouse as described and harvested between 5 and 29 days after anthesis. The blot was probed with the 1kb Sac1 SBEII-1 fragment and subsequently (following blot stripping) with the 1.2kb BamH1 SBEII-2 fragment, both fragments purified and labelled as described. In Figure 12 panel A shows the Ethidium Bromide-stained RNA gel prior to northern transfer. Panel B shows the results of probing with the SBEII-1 probe and panel C shows the results of probing with the SBEII-2 probe. Comparing within and between panels B and C differences can be observed in the relative intensities of the signals at the different time points. In particular a relatively stronger signal intensity is observed with the SBEII-2 probe for the 5 day time point than with the SBEII-1 probe, indicating that the transcript profiles for SBEII-1 and SBEII-2 are distinct, suggesting that the two gene families (SBEII-1 and SBEII-2) are differentially expressed during grain development. The size of the transcripts observed for both SBEII-1 and SBEII-2 is approximately 3.5kb. However the SBEII-2 transcript is slightly smaller than the SBEII-1 transcript.

Plasmid constructions

Standard molecular biology procedures (Sambrook *et al*, 1989) were used for plasmid constructions.

pWxGS+ (Figure 13) comprising a maize granule bound starch synthase gene (Shure *et al* 1983) promoter-GUS-Nos fusion was obtained as a gift to Unilever Research from Sue Wessler (University of Georgia, Athens, USA) and may be obtained on request from that source. The promoter in pWxGS+ is approximately 1.5kb in length and represents a truncated version of a similar, but larger promoter fragment described in Russell & Fromm (1997). The sequence of the promoter (HindIII - BamH1 fragment) in pWxGS+ is presented in Figure 13A (SEQ ID No: 55).

pSRWXGUS1 (Figure 14) was produced by inserting a Sac 1 linker [d(pCGAGCTCG)0] (New England Biolabs [NEB]) (NEB catalogue No 1044) into the SmaI site in pWxGS+.

pVTWXGUS2 (Figure 15) was produced by inserting a BamH1 linker [d(pCGGGATCCCG)] (SEQ ID No: 43) (NEB catalogue No. 1071) into the Ecl136II (an isoschizomer of Sac1 which gives blunt ends) site of pWxGS+

A Sac1 linker was inserted at the XbaI site (which had been blunted using Klenow + dNTps) of the SBEII-1 Clone B6 in the plasmid pT7Blue to produce an intermediate clone. The SBE sequence was then purified from this intermediate clone as a Sac1 fragment and ligated into the Sac1 sites of pSRWXGUS1 replacing the GUS gene sequence to produce the plasmids pSR96-26 and pSR96-29 representing antisense and sense orientations of the SBEII-1 sequence downstream of the Waxy promoter, respectively.

A BamH1 linker was inserted at the XbaI site (which had been blunted using Klenow + dNTps) of the SBEII-2 Clone B11 in pT7Blue to produce an intermediate clone. The SBE sequence was then purified from this intermediate as a BamH1 fragment and inserted into the BamH1 sites of pVTWXGUS2, replacing the GUS gene sequence, to produce the plasmids pVT96-50 and pVT96-53 representing antisense and sense orientations, respectively, of the SBEII-2 sequence downstream of the Waxy promoter.

pVT96-54. A BamH1 linker was inserted at the Xba1 site (which had been blunted using Klenow + dNTPs) of the SBEII-2 clone B9 (equivalent to clone B1) in pT7Blue to produce an intermediate clone. The SBEII-2 sequence was then purified from this

intermediate clone as a BamH1 fragment and inserted into the BamH1 sites of pVTWXGUS2, replacing the GUS gene sequence, to produce the plasmid pVT96-54.

The Waxy-SBE-NOS sequences in the plasmids pSR96-26 and pSR96-29 and pVT96-50 and pVT96-53 were purified as HindIII/EcoRI fragments and inserted into the EcoRI/HindIII sites of plasmid pPBI-97-2 (also known as p97-2) (Figure 16). Plasmid pPBI-97-2 is described in European Patent Application No. 97305694.8 (published as WO 99/06570). Following removal of the ampicillin resistance marker gene the resulting plasmids were designated pSR97-26A- (clone B6 (SBEII-1, sub-class A) in antisense orientation), pSR97-29A- (clone B6 in sense orientation), and pSR97-50A- (clone B11 (SBEII-2, sub-class A) in antisense orientation) and pSR97-53A- (clone B11 in sense orientation) as illustrated in Figures 17, 18, 19 and 20, respectively.

p97-2C (Figure 21) was produced by digesting the polylinker sites Ecl136 II to SmaI in the plasmid pPBI97-2 (Figure 16), ligating and selecting recombinants in which the polylinker region from SmaI to Ecl136 II had reinserted in the opposite orientation.

The Waxy-NOS sequences in pSRWXGUS1 were transferred as a HindIII/EcoRI fragment into the HindIII/EcoRI sites of plasmid p97-2C to produce the plasmid p97-2CWT1 (Figure 22).

pSC98-1 and pSC98-2. The 5' extended SBEII-1 clone 5A1 in pT7Blue (comprising SBE sequence from coordinate 43 to 2003bp in Figure 10) was digested with EcoRI and XbaI, followed by 'in-fill' of overhangs using Klenow polymerase and dNTPs. The resulting blunt ended SBE fragment was gel purified and ligated to p97-2CWT1 (Figure 22) which had been digested with Ecl136II and dephophorylated using calf intestinal phosphatase. The resulting recombinants were screened by restriction digest anaylsis and clones comprising both orientations of the SBE sequence (with respect to the waxy promoter) were identified. pSC98-1 (Figure 23) is an antisense version and pSC98-2 (Figure 24) is a sense version. Following removal of the ampicillin marker gene the resulting plasmids were designated pSC98-1A- and pSC98-2A- respectively.

Ubiquitin promoter - NptII selection construct:pUN1

pUN1 was made in the following way:

A SacI linker was inserted at the SmaI site of the plasmid pAHC25 (Christensen and Quail 1996) to produce an intermediate plasmid. The GUS gene was removed from this intermediate plasmid by digesting with SacI followed by self ligation and identification of recombinant molecules lacking the GUS sequence to produce the plasmid pPBI95-9. pPBI95-9 was digested with EcoRI and following self ligation recombinant molecules lacking the Ubi-BAR sequences were identified. The resulting plasmid is designated pPBI96-23. An NptII sequence was amplified as a PCR product using the primers AG95-7:

5'GATGAGCTCCGTTTCGCATGATTGAACAAGATGG (SEQ ID No: 44) and AG95-8: 5'GTCGAGCTCAGAAGAACTCGTCAAGAAGGC (SEQ ID No: 45), using pPBIBAG3 (Goldsbrough *et al* 1994 as template for the NptII sequence. The amplified product was cloned into the SstI site of pBluescript (Stratagene) and sequenced. The sequencing revealed that the NptII sequence was of the 'mutant' form rather than the wild-type as had been expected. The 'mutant' form carries a single base change which is flanked by unique Nco1 and Sph1 sites. The pBluescript clone was digested with Nco1 and Sph1 to remove the region containing the single base change. Two oligonucleotides, (Npt1:CCCGACGGCGAGGATCTCGTCGTGACC (SEQ ID No: 46) and Npt2: CATGGGTCACGACGAGATCCTCGCCGTCGGGCATG) (SEQ ID No: 47) were then annealed to each other to form an Nco1/Sph1 fragment. This was cloned into the Nco1/Sph1 digested Bluescript/Npt11 clone, and the resulting clone was sequenced to confirm that the gene was now of the wild type form.

The NptII sequences was then purified as a Sac1 fragment and inserted at the SacI site of pPBI96-23 to produce pUN1 (Figure 25). pUN1 includes the wild-type ubiquitin promoter (Ubi promoter), which is also referred to as the ubiquitin regulatory system (abbreviated to URS). The orientation of the NptII sequence in pUN1 was determined by restriction digest analysis. The sequence of the NptII Sac1 fragment is presented in Figure 26 (SEQ ID No: 35).

pUSN99-1 and pUSN99-2. The SBEII-1 (clone B6) sequence was purified as a Sac1 fragment from the plasmid pSR96-26 and inserted at the Sac1 site of pPBI96-23 to produce the plasmids pUSN99-1 and pUSN99-2 (Figures 27 and 28) representing sense and antisense orientations of the SBEII-1 sequences respectively.

pPBI97-2BdUN1. pPBI92-2BdUN1 (also sometimes referred to as p97-2BdUN1) comprises a reconstituted ubiquitin regulatory system (referred to hereafter as a modified ubiquitin promoter or a modified ubiquitin regulatory system (mURS)) which lacks the two overlapping 'consensus heatshock elements' discussed in EP 0342926 and US 5614399. The modified ubiquitin promoter was prepared via PCR amplification of two DNA fragments using maize genomic DNA as template, followed by ligation of the two fragments to produce a single fragment lacking the consensus heatshock (HS) elements. A Kpn1 restriction site was engineered in place of the HS elements. The primers used were designed from sequence information published by Liu et al 1995 (EMBL DNA database accession ZMU29159). To delete the HS elements and to replace with a diagnostic Kpn1 site the ubiquitin promoter and intron sequences were amplified as two fragments using the primer combinations HS1 + Ubi3-3 and HS2 + Ubi5-2, the sequences of which are given below. Primers Ubi5-2 and Ubi3-3 are homologous to sequences in the sequence published by Liu et al 1995. Primers HS1 and HS2 are homologous to sequences located immediately 3' and 5' respectively of the two overlapping HS elements in the ubiquitin promoter as described in EP 0342926 and US 5361399. Both of these primers have a Kpn1 tail at their 5' ends.

Primers

HS1: 5-ATTAGGTACCGGACTTGCTCCGCTGTCGGC - 3 (SEQ ID No: 48)
HS2: 5-TATAGGTACCGAGGCAGCGACAGAGATGCC -3 (SEQ ID No: 49)
Ubi5-2: 5-AGCTGAATCCGGCGGCATGGC -3 (SEQ ID No: 50)
Ubi3-3: 5-TGATAGTCTTGCCAGTCAGGG -3 (SEQ ID No: 51)

The amplified products were subcloned into pGEM TEasy (Promega) to produce the plasmids p97-U1 and p97-U2. The full-length (approx. 2Kb) modified ubiquitin promoter

was reconstructed by subcloning the Kpn1 - Sac1 fragment from p97-U1 into the Kpn1/Sac1 sites of p97-U2 to produce p97-U3. A partial restriction map of the predicted sequence (SEQ ID No: 52) of the cloned fragment in p97-U3 is presented in Figure 29. (The modified ubiquitin promotor (or mURS) is the subject of a copending European Patent Application filed by the present applicants on the same day as the present application, under the reference C1235.01/M). The modified ubiquitin promoter was transferred as a PstI fragment from p97-U3 into plasmid pPBI96-36. The plasmid pBI96-36 (Figure 30) comprises the GUS-Nos reporter gene fusion under the control of the wild-type ubiquitin promoter (derived from pAHC25) in a pUC plasmid backbone. The promoter replaces the wild-type ubiquitin regulatory system in pPBI96-36 to produce an intermediary plasmid p97-dUG1 (Figure 31).

Construction of pPBI97-2BdUN1

The Ubi-Nos sequences in pPBI96-23 were transferred as an EcoRI - HindIII fragment into the EcoRI and HindIII sites of p97-2B (plasmid p97-2B is described in European Patent Application No. 97305694.8 published as WO 99/06570) to produce the plasmid p97-2BUbiNos. The modified ubiquitin promoter was purified as a HindIII/SacI fragment from p97-dUG1 (Figure 31) and transferred into the HindIII and SacI sites of p97-2BUbiNos, replacing the wild-type ubiquitin promoter to produce p97-2BdUbiNos. The NptII sequence in pUN1 was purified as a SacI fragment and transferred into the SacI site of p97-2BdUbiNos to produce pPBI97-2BdUN1 (Figure 32). Following removal of the ampicillin resistance marker using the method as described in WO 99/06570, the resulting plasmid as used for wheat transformation was designated p97-2BdUN1A-

pCaineo

pCaiNeo comprises the NptII gene under control of a CaMV35S promoter and maize Adh1 intron. The plasmid is described in Fromm et al 1986.

Transformation of wheat

The following plasmid combinations (co-bombardments) have been used in the transformation of wheat plants:

Table 2. Plasmid combinations used in wheat transformation experiments.

Starch gene construct/s	Selection marker construct
	pAHC25
pWXGS+	pUN1
pSR97-26A- antisense	pUN1 or p97-2BdUN1
pSR97-29A- sense	p97-2BdUN1 or pCaiNeo
pSC98-1A- antisense	p97-2BdUN1
pUSN-1 sense	p97-2BdUN1
pUSN-2 antisense	p97-2BdUN1
pUSN-1 sense & pUSN-2 antisense	pUN1
pSC98-2A- sense	p97-2BdUN1

The wheat transformation methods used and described here are largely based on those described by Barcelo and Lazzeri, 1995.

Embryo wheat plants of the spring cultivar Bobwhite and the winter cultivar Florida were grown in a glasshouse with 16hr day length supplemented with lights to maintain a minimum light intensity of 500 umol $m^{-2}s^{-1}$ at 0.5M above flag leaf. Glasshouse temperatures were maintained at 19°C+/-1°C during the day and 14°C+/-1°C at night.

Immature embryos of wheat were harvested from developing grain. The seeds were harvested and embryos were cultured at approximately 12 days after anthesis when the embryos were approximately 1mm in length. Seeds were first rinsed in 70% ethanol for 5 minutes and then sterilised in a 10% solution of Domestos bleach (Domestos is a Trade

Mark) for 15 minutes followed by 6 washes with sterile distilled water. Following removal of the embryonic axis the embryos were placed axis surface face down on agargel (Sigma catalogue no. A-3301) solidified MM1 media. The general recipe for MM1 is given in Appendix 1, and the recipes for the various constituents in Appendix 2. The embryos were maintained in darkness for one to two days at 24°C +/-1°C prior to bombardment.

The plasmids pAHC25, pCAiNeo, pUN1 and p97-2BdUN1 were used to provide selection markers in the combinations with starch gene constructs as detailed in Table 2. pAHC25 (Christensen and Quail 1996) contains a chimeric Ubi-BAR gene which provides selection of transformants to phosphinothricin, the active ingredient in herbicides BASTA™ and Bialophos (see Block, M.de. *et al* 1987). The plasmids pCAiNeo (Fromm *et al.*, 1986), pUN1 and p97-2BdUN1 contain chimeric promoter-NptII gene fusions and provide selection of transformants against a range of aminoglycoside antibiotics including kanamycin, neomycin, geneticin and paromycin.

Particle bombardments was used to introduce plasmids into plant cells. The following method was used to precipitate plasmid DNA onto 0.6μm gold particles (BIO-RAD catalogue number 165-2262): A total of 5μg of plasmid DNA was added to a 50μl sonicated for one minute suspension of gold particle (@ 10mg/ml) in a 1.5ml microfuge tube. Following a brief vortex for three seconds 50μl of a 0.5M solution of calcium chloride and 20μl of a 0.05M solution of spermidine free base were added to the opposite sides of the microfuge tube lid. The tube contents were mixed together by closing the lid and tapping the calcium chloride and spermidine to the bottom of the tube. Following a vortex for three seconds the suspension was centrifuged at 13,000 rpm for 5 seconds. The supernatant was then removed and the pellet resuspended in 150μl of absolute ethanol. This requires scraping the gold particles off the inside of the tube using a pipette tip. Following a further three second vortex, the sample was centrifuged again and the pellet resuspended in a total volume of 85μl in absolute ethanol. The particles were vortexed briefly and sonicated for 5 seconds in a Camlab Trisonic T310 water bath sonicator to ensure fine dispersion. An aliquot of 5μl of the DNA coated gold particles were placed in the centre of a macrocarrier (BIO-RAD catalogue no. 115-2335) and allowed to dry for

30 mins. Particle bombardment was performed by using a Biolisitc™ PDS-1000/He (BIO-RAD Instruments, Hercules CA) chamber which is illustrated schematically in Figure 33, using helium pressure of 650 and 900 psi (rupture discs: BIO-RAD catalogue numbers 165-2327 and 165-2328 respectively).

Referring to Figure 33, the illustrated vacuum chamber comprises a housing 10, the inner side walls of which include a series of recesses 12 for receiving shelves such as sample shelf 14 shown at the fourth level down from the top of the housing. A rupture disc 16 is supported in a He pressure shock tube 18 near the top of the housing. A support 20, resting in the second set of recesses 12 down from the top of the housing, carries unit 22 that includes a stopping screen and a number of rings 24, with 11 rings below the support 20 and 3-4 rings above the support 20. Macrocarrier 26 is supported at the top of unit 22. The approximate distance from the rupture disc 16 to the macrocarrier 26 is 25mm, with the approximate distance from the macrocarrier 26 to the stopping screen being 7mm, and the approximate distance from the stopping screen to the sample shelf 14 being 67mm. The top of unit 22 is about 21mm from the bottom of the shock tube 18, and the bottom unit 22 is about 31mm from the top of sample shelf 14.

Immature embryos were bombarded between 1 and 2 days after culture. For bombardment the immature embryos were grouped into a circular area of approximately 1cm in diameter comprising 20-100 embryos, axis side face down on the MM1 media. The Petri dish (not shown) containing the tissue was placed in the chamber on shelf 14, on the fourth shelf level down from the top, as illustrated in Figure 33. The air in the chamber was then evacuated to a vacuum of 28.5 inches of Hg. The macrocarrier 26 was accelerated with a helium shock wave using rupture membranes that burst when the He pressure in the shock tube 18 reaches 650 or 900 psi. Within 1 hour after bombardment the bombarded embryos were plated on MM1 media at 10 embryos per 9cm petri dish and then maintained in constant darkness at 24°C for 2-3 weeks. During this period somatic embryogenic callus was produced on the bombarded embryos.

After 2-3 weeks the embryos were transferred onto agar-solidified regeneration media, known as R media, and incubated under 16hr daylength at 24°C. The general recipe for

R media is given in Appendix 1. Embryos were transferred on fresh plates at 2-3 week intervals. The composition of the regeneration media varied depending on which selection regime was to be used. For transformants bombarded with the BAR gene the 3 amino solution was omitted and PPT (phosphinothricin) at 1mg/L, rising to 3mg/L over a period of three 2-3 week transfers was used for selection. For selection of transformants using the NptII gene three different regimes were used: 1) Geneticin (GIBCO-BRL catalogue no. 10131-019) was incorporated (at 50mg/L) immediately on transfer to regeneration media and maintained at 50mg/L on subsequent transfers to regeneration media. 2) & 3) Embryos were first transferred to regeneration media without selection for 12 days and 2-3 weeks, respectively, and thereafter transferred on to media containing Geneticin at 50mg/L. After 2-3 passages on regeneration media regenerating shoots were transferred to individual culture tubes containing 15 ml of regeneration media at half salt strength with selection at 3mg/L PPT or 35mg/L geneticin depending on whether the BAR gene of NptII gene had been used in the original bombardments. Following root formation the regenerated plants were transferred to soil and the glasshouse.

Genomic DNA isolation and Southern Analyses

Southern analyses of primary transformants and progeny material were carried out as follows: Freeze dried leaf tissues were ground briefly in a Kontes™ pestle and mortar, and genomic DNA extracted as described in Fulton et al, 1995. 5 μg of DNA were digested with an appropriate restriction enzyme according to the manufacturers instructions, and electrophoresed overnight on a 1% agarose gel, after which the gel was then photographed, washed and blotted onto Hybond N+ ™ (Amersham International) according to the method of Southern using standard procedures (Sambrook et al 1989). Following blotting, the filters were air dried, baked at 65°C for 1-2 hours and UV fixed at 312nm for 2 minutes.

Probe preparation and labelling for the Southern analyses of transformed material was carried out as described above.

GUS histochemistry was performed essentially as described in Jefferson (1987).

Evaluation of the ubiquitin promoter for constitutive expression of associated transgenes.

The plasmid pAHC25 (Christensen and Quail, 1996) was transformed into wheat as described in previous sections. Transformants were selected on the basis of resistance to phosphinothricin. Southern blot analyses were carried out on the primary transformants to confirm integration of the plasmid sequences (data not shown). GUS histochemical analyses were also carried out and demonstrated that the ubiquitin promoter is capable of mediating high levels of GUS expression in a range of wheat tissues. Figure 34 A, B, C & D show histochemical localisation of GUS expression in the seed, stem, floral and leaf tissues respectively. Southern blot and GUS histochemical analyses were also carried out on self progeny from primary transformants to confirm that the transformation system used is capable of producing transgenic plants which stably transmit the integrated plasmid sequences to progeny plants. Figure 35 shows a Southern blot of 26 progeny plants of transformant BW119 which had been transformed with pAHC25. In this example genomic DNA from the progeny plants was digested with the restriction enzyme Sac1 and the blot was probed with the GUS gene coding sequence. The Southern blot results are suggestive of the presence of two independently segregating integration loci, each comprising concatamers of pAHC25 plasmid sequences.

Evaluation of the maize waxy promoter for endosperm-specific expression of associated transgenes.

The plasmids pWxGS+ and pUN1 were co-transformed into wheat as described in previous sections. Transformants were selected on the basis of resistance to geneticin. Southern blot analyses were carried out on the primary transformants to confirm integration of the plasmid sequences (data not shown). Gus histochemical analyses were also carried out to determine the expression profile mediated by the maize waxy promoter. The majority of the transformants that expressed GUS exhibited expression specifically in endosperm tissue, demonstrating the suitability of this promoter for mediating endosperm expression of associated transgenes. Figure 36 A & B shows endosperm specific expression of GUS in seeds from two independent transformants. We did not observe GUS expression in pollen grains as was seen by Russell and Fromm (1997), however the

construct they used also incorporated the maize hsp 70 intron which may conceivably have influenced expression both quantitatively and qualitatively.

Transformation of wheat with starch gene constructs.

The various construct combinations detailed in Table 2 were co-transformed into wheat using the procedures as described in previous sections. Transformants were selected on the basis of resistance to geneticin. The primary transformants were confirmed positive by Southern blot analysis. Blots were sequentially probed with an NptII coding sequence probe and a SBE coding region probe. Figure 37 shows an example of a Southern blot which comprises 22 putative transformants which had been co-bombarded with pSR97-29A- or pSR97-26A- and pUN1 or p97-2BdUN1. Genomic DNAs on this blot had been digested with Sac1. The blot was first probed with the NptII probe. Lanes marked with an asterisk correspond to transformants which give a positive signal with the NptII probe. The blot shown in Figure 37 was probed with the SBEII-1 1kb Sac1 fragment. The Sac1 digest is expected to release a 1kb SBEII-1 hybridising band from both pSR97-29A- and pSR97-26A- plasmid sequences, and the intensity of this band will vary depending on the copy number of inserted plasmid sequences. As can be seen in Figure 37 several additional SBEII-1 hybridising bands are also observed. Five of these bands are present in all lanes and result from hybridisation to endogenous wheat SBEII-1 sequences. The additional bands of varying size which are observed in the majority of lanes which show the 1kb hybridising band most likely result from integration events in which one or more copies of the plasmid had been linearised within the 1kb SBEII-1 sequence prior to integration. In the example shown in Figure 37, of the 20 NptII positive plants, 16 were found to be co-transformed with the SBEII-1 sequences, representing a co-transformation efficiency of 80%.

Differential Scanning Calorimetry (DSC)

When heated, an aqueous suspension of starch in excess water undergoes a co-operative endothermic transition known as gelatinisation, as discussed above, entailing a melting of the starch crystallites. Differential scanning calorimetry (DSC) measures the amount of

energy (heat) absorbed or released by a sample as it is heated, cooled or held in a constant (isothermal) temperature. DSC has been widely used to study the gelatinisation and retrogradation of starch.

DSC analyses were carried out on single grains or pools of 5 grains from primary transformants generated through transformation using each of the gene construct combinations detailed in Table 2.

Two different sample preparation and DSC methodologies were used:

Method 1:

Individual seed samples were crushed and ground using a pestle and mortar. The resulting bran was then separated and samples weighed into 50μm aluminium DSC pans. Water, three times by weight, was added and the sample pans sealed. Analyses were performed using a Perkin-Elmer DSC-7 Robotic™ system equipped with an Intercooler II™, for sub-ambient conditions. Samples were heated from 25°C to 80°C at a heating rate of 5°C min^{-1}. Gelatinisation enthalpy, onset and peak and end temperatures were recorded. The thermograms were analysed using the Perkin-Elmer software programs (Thermal Analysis Software 7). Gelatinisation enthalpy is expressed in Joules (J)/gram (g) of sample.

Method 2:

Pools of 5 seeds from a single primary transformant, or single seeds from primary transformants, were milled using a Cemotec 1090™ Sample Mill. The milled sample was then passed through a 250 micron sieve to separate the bran from endosperm. Approximately 5mg of the sieved samples was then accurately weighed into 50μl aluminium DSC pans. Water, three times by weight, was added and the sample pans sealed. Analyses were performed using a Perkin-Elmer Pyris 1™ DSC equipped with autosampler and Intracooler IP. Samples were heated from 40°C to 85°C at a heating rate of 10°C per minute. The thermograms were analysed using the Perkin-Elmer software programs (Pyris Software for Windows v 3.5). Gelatinisation enthalpy, onset and peak

and end temperatures were recorded.

Using method 1, DSC analyses were performed on individual mature grains of primary transformants, transformed with the plasmid combinations pSR97-26A-/pUN1, pSR97-26A-/p97-2BdUN1 and pSR97-29A-/p97-2BdUN1. Data obtained were compared to data from control material which had been transformed with one of the NptII selectable marker plasmids, but did not contain any of the 'starch' plasmids. Table 3 summarises the average onset, peak, end and enthalpy values for the selected material. The majority of samples showed similar values to the control material. However, as can be seen from Table 3 onset, peak and end temperatures were higher for a number of the transgenic samples compared to the control material. For example, transformant BW 326 exhibits a 6.7°C, 4.9°C and 4.6°C increase in onset, peak and end temperatures (respectively) compared to the control sample.

Using method 2 a further series of DSC analyses were carried out on pools of 5 grains from primary transformants, transformed with the plasmid combinations pSC98-1A-/p97-2BdUN1, pUSN-1/p97-2BdUN1, pUSN-2/p97-2BdUN1 and pUSN-1/pUSN-2/pUNI. Data obtained were compared to data from control material which had been transformed with one of the NptII selectable marker plasmids, but did not contain any of the 'starch' plasmids. Table 4 summaries the onset, peak, end and enthalpy values for the selected pooled samples. In many cases there is evidence that the 'starch' transgenic material shows onset, peak and end temperatures which are greater than those observed for the control material. For example, transformant BW727 exhibits a 9.8°C, 8.7°C and 9.1°C increase in onset, peak and end temperatures (respectively) compared to the BW control sample 3, and a 7.6°C, 6.8°C and 7.8°C increase in onset, peak and end temperatures (respectively) compared to the BW control sample 2.

Table 3: Results of DSC analyses on single grains using method 1. Data shown are the averages of between 2 and 6 individual grain samples (T_o, T_p and T_f are onset, peak and end temperatures respectively).

Plasmid combination	Line Code	T_o (°C)	T_p (°C)	T_f (°C)	ΔH (J/g)
BW control sample 1		55.2	59.7	66.5	4.66
pSR97-26A-/pUN1	BW283	57.1	60.4	65.0	2.12
	BW135	57.2	62.1	68.6	4.86
	BW324	57.8	62.1	69.1	5.33
	BW325	58.4	61.8	68.7	3.90
	BW326	61.9	64.6	71.1	2.46
	BW348	60.7	63.4	69.7	3.76
pSR97-26A-/p97-2BdUN1	F227	57.4	61.4	67.3	2.65
pSR97-29A-/p97-2BdUN1	F310	62.1	63.7	69.2	6.75
	F312	59.0	62.3	66.8	1.16
	BW335	56.2	60.8	69.1	4.63
	BW353	59.5	62.7	70.8	3.21
	BW354	55.4	61.7	68.9	4.28
	BW355	57.9	61.5	68.0	3.95
	BW357	55.3	60.6	68.0	3.74
	BW363	56.7	62.5	67.9	1.13
	BW367	59.0	62.5	68.2	2.17
	BW369	57.9	60.9	65.9	1.04
	BW370	53.7	59.4	67.5	6.00
	BW375	57.2	61.5	70.0	4.14
	BW376	54.0	58.1	68.0	3.39
	BW377	53.4	60.9	69.2	2.60
	BW380	54.6	61.6	67.6	2.16
	BW390	56.8	61.2	68.5	1.29
	BW399	57.4	62.7	67.9	1.77
	BW400	60.6	63.6	68.1	0.64
	BW341	51.6	59.0	66.4	1.97

Table 4: Results of DSC analyses on pools of 5 grains using method 2. T_o, T_p and T_f are onset, peak and end temperatures respectively

Plasmid combination	Line Code	T_o (°C)	T_p (°C)	T_f (°C)	ΔH (J/g)
F control sample 1		60.1	63.9	68.0	6.30
BW control sample 2		59.3	64.0	68.4	5.94
BW control sample 3		57.08	62.09	67.08	4.28
pSC98-1A-/p97-2BdUN1	BW449	59.3	62.9	67.9	3.95
	BW477	57.7	63.6	70.6	8.30
	F492	62.3	66.4	70.2	7.60
	F494	63.6	67.3	71.0	5.73
	BW511	59.6	63.8	67.2	0.98
	BW518	60.2	64.9	69.2	3.57
	BW519	58.4	63.6	68.5	4.13
	BW527	58.7	63.7	69.0	6.38
	BW549	59.9	64.8	69.3	4.48
	BW550	60.2	64.6	68.9	5.06
	BW552	60.8	62.9	67.9	3.74
	BW553	59.5	63.9	67.5	3.60
	BW555	61.0	66.1	68.2	5.43
	BW557	62.7	66.9	71.0	5.08
	BW559	61.6	65.9	70.8	5.08
	BW563	61.4	65.1	69.4	1.90
	BW564	59.4	64.5	73.2	7.08
	BW576	61.8	65.6	69.3	2.65
	BW587	61.3	65.4	69.4	5.36
	BW614	63.9	67.9	71.8	5.83

	BW618	61.3	65.6	69.7	3.54
	BW583a	58.9	63.7	68.0	3.54
	BW631	61.5	65.6	69.7	4.52
	BW633	61.9	66.0	70.2	5.12
	BW634a	60.8	64.9	70.2	5.10
	BW637a	62.8	67.2	72.0	5.16
	BW639	61.8	65.1	68.9	2.15
	BW640a	62.2	66.7	71.0	3.23
	BW642	63.2	67.2	70.9	4.90
	BW698	62.9	67.0	70.9	4.48
	BW700a	63.8	67.6	71.2	3.41
	BE524a	59.4	64.3	68.9	4.05
pUSN-1/p97-2BdUN1	BW622	59.0	64.1	68.7	4.32
	BW628	56.2	63.3	66.0	6.09
	BW645	57.5	65.6	69.5	5.97
	BW646	61.6	66.4	67.7	3.99
	BW647	61.3	65.4	69.0	3.47
	BW648	59.8	64.4	68.8	4.65
	BW649	61.3	65.6	70.1	5.07
	BW656	59.9	64.6	69.2	5.38
	BW660	62.0	67.3	71.0	4.23
	BW661	61.5	65.8	69.6	3.88
	BW664	61.1	66.1	70.8	4.81
	BW665	61.6	66.5	69.4	5.25
	BW667	63.0	67.1	70.8	3.91
	BW672	63.0	68.1	71.9	5.43
	BW673A	63.1	67.7	71.6	4.83
	BW675	62.1	66.4	71.3	10.97

	BW676	59.8	67.3	71.2	4.21
	BW678	63.0	66.3	69.3	1.20
	BW680	60.8	65.3	70.1	4.94
	BW701	62.3	67.5	72.2	4.70
	BW706	63.0	67.3	71.3	4.94
	BW707	60.9	65.8	70.0	4.77
	BW708	61.7	65.5	68.8	6.11
	BW726	62.6	67.5	71.3	5.44
	BW755	60.8	65.8	70.6	5.18
	BW702	61.9	67.0	71.0	4.44
	BW756	62.3	66.1	69.7	4.83
pUSN-2/p97-2BdUN1	BW625	62.7	68.2	73.8	4.27
	BW653	60.4	65.3	70.1	6.52
	BW704	60.9	66.2	70.2	4.19
	BW718	61.3	66.9	71.2	4.15
	BW719	62.2	67.2	71.7	5.32
	BW722	64.8	67.5	70.0	2.14
	BW740	63.4	67.9	72.3	5.67
	BW741	62.6	66.9	70.5	5.30
	BW742	64.6	67.9	72.0	6.66
	BW752	62.3	66.3	70.0	4.63
pUSN-1/pUSN-2/pUN1	BW685	62.6	65.5	69.0	2.60
	BW686A	61.9	66.3	70.2	4.45
	BW714	63.0	67.6	71.3	3.53
	BW727	66.9	70.8	76.2	5.19
	BW728	62.0	66.3	70.4	5.70
	BW731	63.3	67.9	73.0	4.90

	BW732	63.5	66.8	70.8	4.11
	BW748	62.1	67.4	71.9	5.38
	BW794	62.8	67.5	71.8	5.17

Appendix 1.

Recipe for 2x concentrated MM1 media

Constituent	**Volume of stock per litre of 2x concentrated media**
Macrosalts MS (10X stock)	200ml
Microsalts L (1000x stock)	2ml
FeNaEDTA MS (100x stock) [Sigma catalogue F-0518]	20ml
Modified Vits MS (x1000)	1ml
3 amino acid solution (25x stock)	40ml
myo inositol (Sigma catalogue number I-3011)	0.2g
sucrose	180g
$AgNO_3$ (20mg/ml stock) Added after filter sterilisation	1ml
Picloram (1m/ml stock) Added after filter sterilisation	4ml

Filter sterilise and add to an equal volume of moulten 2x agargel (10g/L).

Recipe for 2x concentrated R media

Constituent	**Volume of stock per litre of 2x concentrated media**
Macrosalts L7 (10X stock)	200ml
Microsalts L (1000x stock)	2ml
FeNaEDTA MS (100x stock)	20ml
Vits/Inositol L2 (200x stock)	10ml
3 amino acid solution (25x stock)	40ml
Maltose	60g
2,4-D (1mg/ml stock) added after filter sterilisation	200μl
Zeatin cis trans mixed isomers (Melford labs catalogue no. Z-0917) (5mg/ml stock) added after filter sterilisation	2ml

Filter sterilise and add to an equal volume of moulten 2x agar (16g/litre)

Appendix 2

Recipes for constituents of MM1 and R media

Microsalts L (1000x stock)

	per 100ml
$MnSO_4.7H_2O$	1.34g
H_3BO_3	0.5g
$ZnSO_4.7H_2O$	0.75g
KI	75mg
$Na_2MoO_4.2H_2O$	25mg
$CuSO_4.5H_2O$	2.5mg
$CoCl_2.6H_2O$	2.5mg

Filter sterilise through a 22μm membrane filter

Store at 4°C

Macrosalts MS (10X stock)

	per litre
NH_4NO_3	16.5g
KNO_3	19.0g
KH_2PO_4	1.7g
$MgSO_4.7H_2O$	3.7g
$CaCl_2.2H_2O$	4.4g

NB: Dissolve $CaCl_2$ before mixing with other components

NB: Make up KH_2PO_4 separately in sterile H_20, and add last.

Store solution at 4°C after autoclaving

Modified MS Vits (1000x stock)

	Per 100ml
Thiamine HCl	10mg
Pyridoxine HCl	50mg
Nicotinic acid	50mg

Store solution in 10ml aliquots at -20°C

3 amino acid solution (25x stock)

	Per litre
L-Glutamine	18.75g
L-Proline	3.75g
L-Asparagine	2.5g

Store solution in 40ml aliquots at -20°C

Macrosalts L7 (10x stock)

	per litre
NH_4NO_3	2.5g
KNO_3	15.0g
KH_2PO_4	2.0g
$MgSO_4.7H_2O$	3.5g
$CaCl_2.2H_2O$	4.5g

NB: Dissolve $CaCl_2$ before mixing with other components

NB: Make up KH_2PO_4 separately in 50ml H_20 and add last

Store solution at 4°C after autoclaving

Vits/Inositol (200x stock)

200x Stock	Per 100ml
Inositol	4.0g
Thiamine HCl	0.2g
Pyridoxine HCl	0.02g
Nicotinic acid	0.02g
Ca-pantothenate	0.02g
Ascorbic acid	0.02g

Store solution in 40ml aliquots at -20°C

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